Diagnosis and immunophenotype of 188 pediatric lymphoblastic lymphomas treated within a randomized prospective trial: experiences and preliminary recommendations from the European childhood lymphoma pathology panel

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Abstract: The majority of lymphoblastic (precursor cell) neoplasms presents as leukemias. Consequently, the guidelines for lineage determination and subtyping of precursor cell neoplasms were primarily established for flow cytometry methods. Large-scale studies of nonleukemic lymphoblastic lymphomas are lacking so far. We analyzed a large series of pediatric patients with lymphoblastic lymphoma treated within a prospective randomized trial (the Euro-LB 02 study). Among 193 lymphomas, in which a detailed immunohistochemical analysis was carried out, there were several unusual and diagnostically challenging morphologic and immunophenotypical variants. These included 11 lymphomas with mixed phenotypes expressing markers of at least 2 hematopoietic lineages, 7 terminal deoxynucleotide transferase-negative lymphoblastic lymphomas, and 3 undifferentiated hematopoietic neoplasms that could not be assigned to any lineage with certainty. Our data indicate that World Health Organization guidelines for lineage determination and subtyping of precursor cell leukemia need to be adapted before they can be applied to immunohistochemical diagnosis of lymphoma. Using the experience from this cohort we suggest a resource-saving diagnostic staining panel for the immunohistochemical analysis of precursor cell neoplasms in formalin-fixed paraffin-embedded tissue.

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Diagnosis and Immunophenotype of 188 Pediatric Lymphoblastic Lymphomas Treated Within a Randomized Prospective Trial: Experiences and Preliminary Recommendations from the European Childhood Lymphoma Pathology Panel.

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Abstract

The majority of lymphoblastic (precursor cell) neoplasms present as leukemias. Consequently, the guidelines for lineage determination and subtyping of precursor cell neoplasms were primarily established for flow cytometry methods. Large scale studies of non-leukemic lymphoblastic lymphomas are lacking so far. We analyzed a large series of pediatric lymphoblastic lymphoma patients treated within a prospective randomized trial. (the Euro-LB 02 study). Among 193 lymphomas, in which a detailed immunohistochemical analysis was performed, there were several unusual and diagnostically challenging morphological and immunophenotypical variants. These included 11 lymphomas with mixed phenotypes expressing markers of at least two hematopoietic lineages, 7 TdT negative lymphoblastic lymphomas and 3 undifferentiated hematopoetic neoplasms that could not be assigned to any lineage with certainty. Our data indicate that WHO guidelines for lineage determination and subtyping of precursor cell leukemia need to be adapted before they can be applied to immunohistochemical diagnosis of lymphoma. Using the experience from this cohort we suggest a resource-saving diagnostic staining panel for the immunohistochemical analysis of precursor cell neoplasms in formalin-fixed paraffin-embedded tissue.

Introduction

Lymphoblastic (precursor) lymphoma is the second most common type of non-Hodgkin lymphoma in children and adolescents in Western Europe [6] and derives from immature precursor lymphoblasts whose differentiation is arrested at early stages of maturation. Treatment trials in children and adolescents subdivide lymphoblastic neoplasms into two diseases: acute lymphoblastic leukemia (ALL) and lymphoblastic lymphoma (LBL) [19]. There is an ongoing discussion as to whether LBL and ALL are two distinct entities or whether they represent two variants of one and the same disease and the distinction is arbitrary [6]. The extent of blast infiltration of the bone marrow is currently used to distinguish between the two diseases, with 25% of tumor cells in the smears as an arbitrarily chosen cut-off [26]. LBL frequently presents as a mediastinal tumor in patients who are often critically ill but have little or no bone marrow infiltration [19]. Thus, diagnostic material obtained in cases of LBL is often limited to small specimens. Consequently, most of our knowledge about lymphoblastic neoplasms derives from cases of ALL and the number of studies on LBL is limited [6, 8, 11, 16-18, 22] . This situation is reflected in the WHO2008 classification, which refines the diagnostic criteria for precursor cell neoplasms in a way that might pose a problem for pathologists who have to deal with tissue biopsy specimens without access to multicolor flow cytometry (FC) [26].

This study represents the largest published series of LBL cases characterized by immunophenotyping. All samples were obtained during a prospectively randomized clinical trial for children and young adults under the age of 21 (the Euro-LB 02 study). The analysis was conducted on formalin-fixed paraffin-embedded material (FFPE) and its purpose was to define the immunophenotype of LBL in children. The diagnostic staining approach for LBL recommended here was developed for use on FFPE specimens and for widespread application, including general pathology laboratories, and is intended as a step-wise and material-sparing diagnostic process. Thus, this is not a competing but a complementary concept to that of the WHO.

Material and Methods

Patients and materials:

The patient cohort was recruited in a prospective randomized trial, the Euro-LB 02 study ("Euro-LB 02 treatment protocol for lymphoblastic lymphomas of the European Intergroup Cooperation on Childhood Non-Hodgkin Lymphoma (EICNHL) International Multicentre Therapy Study Group"), which included patients suffering from lymphoblastic lymphoma (LBL) who were 21 years and younger. According to current criteria, LBL patients had less that 25% blasts in the bone marrow smears.

During the time of recruitment (2002 – 2008) patients were registered by 8 participating study groups in 14 European countries. All cases, in which the initial diagnosis was made on tissue biopsy specimens (n=279) were identified by the international data base at the NHL-BFM study center in Giessen, Germany. Of these the national reference pathologists were able to collect 196 for review. In 83 patients no reference pathology review was possible because the diagnosis had been based on liquid material rather than a tissue biopsy (n=8), because of a lack of access to the biopsy material for central review (n=70) or insufficient material for appropriate review (n= 5).

Pathology review and immunophenotype

Selected cases were reviewed by the panel of national reference pathologists at a multiheaded microscope, including all challenging cases (TdT-negative, ambiguous lineage, unusual morphology, staining difficult to interpret, low quality specimen). In addition, randomly chosen cases from every participating study group were reviewed to ensure that the quality of the immunohistochemical staining and interpretation adhered to the defined standards.

All immunohistochemical (IHC) stainings were performed on whole tissue sections except for a few supplemental stains that were performed centrally on tissue microarrays (n=55, n=3 and n=54 for Pax5, CD1a and CD19, respectively). The scoring procedure was agreed upon at a consensus meeting and all the stainings were scored semiquantitatively by individual reference pathologists as negative, weak (<30% positive tumor cells or all tumor cells weakly positive), positive (>30% positive tumor cells) or not interpretable. The staining panel included CD20, CD79a, Pax5, CD19, CD10, IgM, CD3, CD1a, CD4, CD8, CD5, CD2 MPO, TdT, and CD34. Due to the retrospective nature of the study, the staining procedures and antibody sources for these markers varied between the participating countries and over time. Since biopsy material obtained from the mediastinal tumors was often limited, the number of performed immunohistochemical stainings also varied from case to case.

A T-cell lineage was established on the basis of CD3 expression, a B-cell lineage by the expression of at least two of the following B-cell markers, CD19, CD79a, Pax5 and/or CD20, and a myeloid lineage by expression of MPO. Mixed lineage acute leukemia (MPAL) was diagnosed if the tumor cells fulfilled the diagnostic criteria for two lineages (figure 4). In accordance with the WHO classification, “bi-lineal” and “bi-phenotypical” diseases were not distinguished. Undifferentiated hematopoietic neoplasm (UHN) was diagnosed if a lineage could not be determined according to the above mentioned criteria and after exclusion of other non-hematopoietic small round cell tumors.

Statistical analysis

Fisher’s exact test was performed using GraphPad Prism™ software.

Results

Diagnosis

Since the clinical analysis of the Euro-LB 02 trial is not as yet available this study focuses on the pathology review. The clinical results will be published elsewhere. Overall data for 196 study patients were collected and reviewed by the panel. In eight of the registered patients the panel excluded the diagnosis of precursor cell lymphoma (3 diffuse large B-cell lymphomas, 1 Burkitt lymphoma, 2 mature aggressive B-cell lymphomas, and 2 unclassifiable lymphomas due to insufficient immunostains and these cases were excluded from all further analysis. In the remaining cohort (n=188), the diagnostic categories of the WHO classification for precursor cell neoplasms were used (Swerdlow et al., 2008). The vast majority of LBL displayed a typical morphology with monomorphic sheets of medium-sized blasts with round nuclei, finely dispersed nuclear chromatin and a narrow rim of cytoplasm. However, in a small subset of cases (n=3) we observed considerable morphological variation (figure 1).

In 146 cases all stainings required for application of the criteria outlined in the Material and Methods section were evaluated. Of those 42 (29%) were classified as precursor B-cell lymphoblastic lymphoma (B-LBL), 90 (62%) as precursor T-cell lymphoblastic lymphoma (T-LBL), 11 (7%) as MPAL and 3 (2%) as UHN. A subset of confirmed precursor lymphomas (n= 42) displayed markers of at least one lineage (36% B, 64% T), but the IHC panel was not complete to exclude any additional lineage differentiation.

T-cell lymphoblastic lymphoma (T-LBL)

Within the group of 90 T-LBL, we initially attempted to distinguish the immunological maturation stages: pro-T-LBL, pre-T-LBL, cortical T-LBL and medullary/mature T-LBL as defined by the EGIL classification [2]. To apply this classification it is necessary to distinguish between cytoplasmic and membranous CD3 staining for differentiation of early pro- and pre-T-LBL and medullary/mature T-LBL. However, the panel agreed that a distinction between cytoplasmic and membranous CD3 staining can not be reliably made using IHC on FFPE specimens (figure 2). Thus, we could only distinguish 49 cortical T-LBL (54%), which differed from all other subtypes in their expression of CD1a, and noncortical (n=35, 39%) or unclassifiable T-LBL that lacked sufficient IHC stainings for subtyping (n=6, 7%). This percentage of cortical LBL in our study was comparable to previous publications [6].

In order to evaluate whether additional IHC stainings might help to distinguish early T-LBL (pro- and pre-T-LBL) from later T-LBL (cortical and medullary/mature T-LBL) we assessed

possible markers of early stages of T-cell differentiation (CD34, CD10, CD4/CD8 double negative) in cortical and noncortical T-LBL. CD34 and CD10 were expressed in a comparable fractions of cases in both the cortical and noncortical subtypes (12% versus 23%, p=0.3386 and 67% versus 56%, p=0.5314, respectively, Table 1). Cases expressing either CD4 or CD8 (single positive) were significantly more frequent in the group of noncortical T-LBL (42%) than in the group of cortical T-LBL (18%, p=0.0354). As expected, cortical T-LBL were more often double positive for CD4 and CD8 than noncortical T-LBL (78% versus 36%, p=0.0003) and showed a lower frequency of double negativity (4% versus 23%, p=0.284) (table 1).

B-cell lymphoblastic lymphoma (B-LBL)

Subtyping of B-LBL was performed according to the well established categories of CD10-/IgM- pro-B-LBL (0/42, 0%), CD10+/IgM- common-B-LBL (20/42, 46%) and CD10+/IgM+ pre-B-LBL (5/42, 12%) [2]. Lymphomas, in which the B-cell nature was established but that lacked the necessary data for subtyping were categorized as not otherwise specified (nos). Within the group of B-LBL nos (n=17, 41%), 15 (36%) were CD10 positive and either common- or pre-B-LBL, but lacked reliable IgM staining. When only the 25 B-LBL with complete subtyping were considered, there was no difference in the subtype distribution in our series as compared with a previously published large series of precursor pB-ALL (data not shown) [21].

The majority of B-LBL were positive for CD79a. However, since CD79a might be expressed in a considerable number of T-LBL and in accordance with the criteria of the WHO classification, we required a second positive B-cell marker in order to prove B-cell lineage differentiation., Also we determined the sensitivity and the specificity of B-cell markers in B-LBL. 12% of T-LBL samples (3/47, cortical and 6/33, noncortical, Table 2) were positive for CD79a, but none for any of the other B-cell markers (CD20, CD19 and Pax5). It should be noted that in CD79a positive T-LBL we also observed CD10 expression in 4/6 cases. Whereas the sensitivity of CD20 for the detection of the B-cell lineage in pLBL is poor (12/35, 34% positive), the marker Pax5, was highly sensitive, with 35/35 (100%) of the analyzed B-LBL cases being positive.

Mixed lineage acute leukemia/lymphoma (MPAL)

The group of MPAL consisted of 7 lymphomas with myeloid and B-cell lineage markers, 2 with myeloid and T-cell lineage and 2 with B-cell and T-cell lineage (table 3, figure 3). All MPAL were positive for TdT but they expressed CD34 in only 5 of 10 cases that could be evaluated. All T-lineage MPAL were negative for CD1a (0/4) and CD56 (0/4).

Undifferentiated hematological neoplasia (UHN)

Three cases were classified as UHN because they lacked any lineage specific markers but expressed markers of immature hematopoetic cells such as TdT (1/3) or CD34 (2/3). Furthermore, less specific hematopoietic markers were detected (table 4) and other small round cell tumors were excluded, e.g. by staining for CD56 (0/3, data not shown). Cases number 1 and 3 presented as localized bone tumors in the lumbar spine and the iliac bone, respectively, whereas case number 2 presented as a mediastinal tumor. Case number 3 was initially misdiagnosed as Ewing sarcoma because of its strong CD99 positivity in the absence of other main lineage markers and its suggestive localization within the bone.

TdT negative LBL

Terminal deoxynucleotide transferase (TdT), the major marker of a precursor cell neoplasm in the B- and T-cell lineage, was expressed in 166/173 (96%) of precursor cell neoplasms. Seven LBL were diagnosed as TdT negative LBL, but all of these lymphomas displayed the typical morphology of LBL in addition to the immunophenotypic features of precursor cell neoplasms, such as weak or partial expression of TdT in 1-5% of the lymphoma cells (4/7), expression of CD34 (1/7), coexpression of CD3 and CD79a (1/7), or coexpression of CD4 and CD8 (1/7).

Discussion

The new WHO lymphoma classification published in 2008 [25] introduced several important changes in the field of B- and T-precursor cell neoplasms: (i) the marker definition for determining the cell lineage was changed and the EGIL scoring system for biphenotypic leukemia [2] was abandoned, (ii) the terminology and definition of mixed phenotype precursor cell neoplasms was established and (iii) subtypes defined by recurrent genetic aberrations were introduced. Most of our knowledge on the immunophenotypes of precursor cell neoplasms has been gathered from cases of leukemic disease by means of FC. Several of the current diagnostic criteria are difficult to apply on tissue specimens. The advantage of our study is that it points out the specific problems associated with the diagnosis of precursor cell neoplasms by means of histology and IHC on FFPE material. Biopsy material and consequently the number of feasible immunostains are often limited due to localization of the tumors. Therefore we propose a well reflected algorithm (figure 5) applicable for diagnosing and subtyping LBLs in daily practice.Using this algorithm, we identified T-LBL twice as often as B-LBL, which confirms previous reports [6].

The diagnosis of LBL should combine (i) typical morphology, (ii) proof of precursor cell immunophenotype, (iii) precise lineage definition and (vi) subtyping by additional stainings and/or genetic analysis to obtain a final diagnosis. In our data TdT expression is the most useful tool for confirming the precursor cell nature of a lymphoma. It should be used to stain all cases of LBL as well as other blastic neoplasms in children, since morphological variants of LBL may mimic other aggressive lymphomas, such as diffuse large B-cell lymphomas (figure 1). Moreover, this staining can be helpful in the differential diagnosis of immature hematological neoplasms versus other small round blue cell tumors, but it should be noted that rare nonhematopoietic tumors such as Merkel cell carcinomas may also express TdT [3, 5, 24] . In addition TdT-positive bone marrow hematogones have to be considered when judging trephine specimens [12]. Similarly TdT-positive cortical thymocytes of normal thymus should not be mistaken for T-LBL in small mediastinal biopsies. IHC staining for cytokeratin can be of help in such situations. In the rare cases of TdT negative LBL with otherwise typical lymphoblastic morphology, either expression of CD1a or CD34, coexpression of CD79a and CD3 or coexpression of CD4 and CD8 can be used to identify the precursor cell nature of the lymphoma. Coexpression of CD4 and CD8 has also been reported in cases of mature T-NHL, such as T-cell prolymphocytic leukemia and adult T-cell leukemia/lymphoma [26]. However these entities are usually not a differential diagnosis in children.

Immunohistochemical stainings for CD3 and myeloperoxidase (MPO) are powerful tools for identifying the T-cell and myeloid lineage with a single staining for each lineage, according to the requirements of the WHO 2008 classification (figure 4). Proof of the B-cell lineage requires at least two B-cell markers to be positive. We chose CD79a staining as primary screening marker for two reasons. First, this is a widely known pan-B-cell marker that is accepted by the WHO and is also available in general pathology laboratories for rapid initial diagnosis in clinically urgent situations. Second, it is - as we showed in our series - a highly sensitive marker for detecting B-cell differentiation in LBLs and MPALs. Nevertheless lineage assessment by means of CD79a alone would be hampered by its relatively low specificity, given the substantial proportion of CD79a co-expressing T-LBLs (12% in our series). Combination with a second B-cell marker is therefore mandatory. CD19 was found in our series to be a highly sensitive and specific B-cell marker. Pax5, a nuclear transcription factor involved in B-cell development [7], has not yet been recommended by the WHO for lineage determination in ALL. Our data confirm very recent observations that Pax5 is a highly specific and sensitive B-cell marker within the context of LBLs [15]. It has nevertheless to be remembered that Pax5 has also been reported in rare non-hematopoetic neoplasms including tumors of the small round blue cell category [13, 23].

We thus recommend screening all cases of LBL with CD3, MPO and CD79a and adding CD19 or Pax5 in CD79a positive cases to confirm or rule out B-cell lineage differentiation (figure 5). The use of CD20 in the lineage determination of precursor cell neoplasms is of limited value, since the sensitivity of this marker is low in B-LBL. Recently, CD22 staining protocols for FFPE specimens were published and it may prove to be another useful marker for confirming B-cell lineage [4]. Because T-LBL cases may coexpress CD79a and CD10, CD10 might be useful as a second marker for confirming B-cell lineage only in CD19 strongly positive B-LBL, as recommended by the WHO classification [26], but if CD79a was applied, the second line B-cell marker should be either Pax5 or CD19 (Figure 5). It should also be noted that a precise quantification of the staining intensity of immune markers, as required for CD10 along with CD19 in the MPAL criteria of the WHO classification, cannot be reliably applied for immunohistochemistry.

Although the criteria for lineage determination are now well defined by the WHO classification, immunophenotypical subtyping of stage of differentiation in B- and T-LBL is still based on the EGIL criteria and widely used in ALL [2]. However, we faced several problems when we applied the proposed scheme for IHC staining on FFPE specimens. The subtyping of B-LBL is based on the expression of IgM and CD10. This can cause difficulties in cases where reliable IgM staining can not be achieved. Nevertheless, subtyping into the well established categories used for ALL, namely CD10-/IgM- pro-, CD10+/IgM- common- and

CD10+/IgM+ pre-B-LBL [2] was in our study possible in B-LBL when biopsy specimens of appropriate size are available It should be stressed that TdT positive pLBL very rarely express surface immunoglobulin light chains [10].

The problems of subtyping according to the stage of differentiation are more pronounced in T-LBL, where it is necessary to differentiate between cytoplasmic and membranous CD3 staining to distinguish pro- and pre-T-LBL from medullary/mature T-LBL [2]. Although this is a standard procedure in flow cytometry, we did not find this distinction feasible for immunohistochemistry on FFPE specimens. Nevertheless, the cortical subtype could be reliably differentiated from noncortical T-LBL on the basis of CD1a expression, a distinction that has been found clinically relevant in ALL [6]. Therefore we suggest using CD1a staining to identify cortical T-LBL in daily practice and lumping all further subtypes (pro/pre- and medullary/mature) together as noncortical T-LBL (Figure 5). It remains questionable whether the immunophenotypic subtypes of T-LBL defined by the EGIL criteria reflect a possible "cell of origin" accurately, since antigen expression in lymphomas may not precisely reflect the antigen expression pattern during during T-cell development [20]. Being aware of the plasticicity in thymic developement it is generally considered that CD8+/CD4- single positive T-LBL should derive from relatively mature thymocytes, but we found CD34 expression, a marker indicating early T-cell precursors, in two of six CD8+/CD4- lymphomas analyzed (data not shown). Also, unexpectedly, CD34 and CD10 expression was seen in roughly the same percentage in cortical and noncortical T-LBLs (table 1). Genetic analysis of T-cell receptor gene rearrangement, flow cytometry data and results of molecular profiling can be expected to help identify new markers for distinguishing biologically relevant subtypes of T-LBLs in the future. Moreover the possible clinical significance of CD34 expression in T-LBL should be evaluated in future studies [27]. Further new diagnostic markers might be obtained from molecular profiling, although the published series are rather small [11, 18].

Our study has several limitations. First of all, no genetic analysis was performed. Since the WHO classification uses several recurrent aberrations to delineate subtypes of B-ALL and MPAL [26] [14], future studies on the cohort presented here will be valuable for studying these aberrations in LBL. Second, no clinical correlation could be performed, since the results of Euro-LB 02 trial have not been analyzed as yet. Our cohort will be the basis for future studies investigating prognostic markers that have been described in T-ALL for their applicability in T-LBL [28] and for understanding the clinical relevance of multi-lineage differentiation in neoplasms that present clinically not as ALL [1] but as lymphomas.

We did not screen systematically for monocytic lineage differentiation, as recommended by the WHO. As discussed above, the WHO criteria were primarily designed for leukemias. Monocytic differentiation occurs frequently in AML, but may prove of lesser importance in

diseases presenting as lymphomas. Nevertheless, we identified an unexpectedly large number of precursor cell neoplasms with mixed lineage marker expression within our cohort of lymphomas that were diagnosed and included in the trial primarily as lymphoblastic lymphomas (7%). In these MPAL, myeloid/B-cell differentiation seemed to be the most frequent phenotype, a finding that is comparable to data published for leukemias (Xu et al., 2009) (Bene, 2009). 5/10 cases tested were also positive for CD34. Unfortunately, we do not have the cytogenetic data on these cases and therefore cannot exclude the possibility of myelosarcoma with t(8;21) in some of these cases. The prognostic relevance of this finding is still uncertain. However, MPAL presenting as leukemia have been reported to show a very aggressive clinical course in adults [29] and also behaves more aggressiv than pALL in children [9]. Our data indicate that systematic staining of precursor cell neoplasms for all three lineages (B-cell, T-cell and myeloid) should be performed in every case even if a lineage differentiation has already been assigned to the disease by a limited staining panel. The staining algorithm that we propose for pediatric LBL will help to limit immunohistochemical stainings and to identify MPAL (figure 5). In the very rare cases of UHN that we observed nonhematopoietic small round cell tumors should be carefully excluded and the staining panel for hematopoietic markers must be extended. It should be noted that CD99, a marker frequently used to identify Ewing's sarcoma, may also be expressed in hematopoietic precursor cell neoplasms. Expression of CD56 in absence of other haematopoietic markers in tumors of the posterior mediastinum neuroblastoma has to be considered in the differential diagnosis.

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Figure Legends

Figure 1: Examples of typical and unusual morphology in LBL. Typical morphology LBL (A: H&E, B: TdT). Mediastinal biopsy specimen from a 3-year old male patient with T-LBL (noncortical subtype) showing an unusual immunoblastic morphology (C: H&E, D: TdT)). Lymph node biopsy specimen from a 6-year-old male with a B-LBL (common subtype) with unusual pleomorphic, centroblastic morphology (E: H&E, F: TdT). Original magnification 400x, inserts 1000x.

Figure 2: Immunohistochemistry for CD3 in two cases of T-LBL whose CD3 expression pattern had been determined by flow cytometry as cytoplasmic (A) and membranous (B). The corresponding immunohistochemical staining is shown.

Figure 3: Mixed phenotype acute leukemia/lymphoma expressing CD3, CD5, CD19, CD79a but no Pax5. MPO in this case was negative.

Figure 4: Requirements for assigning a lineage differentiation according to the WHO classification, which was designed primarily for flow cytometry (Swerdlow et al., 2008).

Figure 5: Recommended staining algorithm for pediatric LBL for immunohistochemistry on FFPE specimens.